Molecular response of **Sargassum vulgare** to acidification at volcanic CO_2 vents : insights from de novo transcriptomic analysis

Molecular response of *Sargassum vulgare* to acidification at volcanic CO_2 vents - insights from *de novo* transcriptomic analysis

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Running title: *Sargassum vulgare* response to acidification

Abstract

Ocean acidification is an emerging problem that is expected to impact ocean species to varying degrees. Currently, little is known about its effect on molecular mechanisms induced in fleshy macroalgae. To elucidate genome wide responses to acidification, a comparative transcriptome analysis was carried out between *Sargassum vulgare* populations growing under acidified conditions at volcanic CO_2 vents and a control site. Several transcripts involved in a wide range of cellular and metabolic processes were differentially expressed. No drastic changes were observed in the carbon acquisition processes and RuBisCO level. Moreover, relatively few stress genes, including those for antioxidant enzymes and heat shock proteins, were affected. Instead, increased expression of transcripts involved in energy metabolism, photosynthetic processes, and ion homeostasis suggested that algae increased energy production to maintain ion-homeostasis and other cellular processes. Also, an increased allocation of carbon to cell wall and carbon storage was observed. A number of genes encoding proteins involved in cellular signaling, information storage and processing, and transposition were differentially expressed between the two conditions. The transcriptional changes of key enzymes were largely confirmed by enzymatic activity measurements. Altogether, the changes induced by acidification indicate an adaptation of growth and development of *S. vulgare* at the volcanic CO_2 vents, suggesting that this fleshy alga exhibits a high plasticity to low pH and can adopt molecular strategies to grow also in future more acidified waters.

45

46 ## Introduction

47 The anthropogenic emission of carbon dioxide (CO_2) in the atmosphere has increased in
48 the post industrialization era and it is still accelerating. Over the last 200 years, oceans have
49 absorbed a major portion of these emissions, acting as an efficient sink for CO_2 (Sabine *et al.*
50 2004). However, current CO_2 emission rates exceed the buffering capacity of the oceans and
51 cause a shift of marine carbonate chemistry and a decrease of pH. Consequently, oceanic pH,
52 which has already decreased 0.1 units since last century, might face a drop of extra 0.3-0.5 units
53 by the end of this century, a phenomenon known as ocean acidification (OA; Caldeira & Wickett
54 2005).

55 A wide range of marine organisms is affected by OA: calcifiers are more sensitive than
56 non-calcifying organisms because the reduction of the saturation state of $CaCO_3$ in seawater
57 causes faster dissolution of their skeletons (Kroeker *et al.* 2010). Therefore, the effect of OA may
58 widely affect ocean biology, causing a shift in the community structure and population dynamics
59 (Porzio *et al.* 2011; Kroeker *et al.* 2013; Harvey *et al.* 2014). Besides calcification, OA can
60 interfere with other processes such as photosynthesis. Theoretically, increased CO_2 availability
61 should benefit the growth and primary production of marine plants (Koch *et al.* 2013).
62 Therefore, marine autotrophs which rely solely on aqueous CO_2 or having less efficient carbon
63 concentrating mechanisms (CCM) should benefit from OA. The macroalgae with CCM could
64 also take advantage if they shut down energy requiring active bicarbonate transport and support
65 passive CO_2 diffusion for carbon acquisition (Wu *et al.* 2008). Conflicting results have been
66 reported so far, when short and long-term effects of elevated CO_2 on macroalgae were tested

either separately or in combination with other stressors in the laboratory conditions. For example, among algae belonging to the *Sargassaceae*, photosynthesis was stimulated by short-term exposure to high CO_2 levels in *Hizikia fusiformis*, while it was inhibited in the germlings of *Sargassum henslowianum*, under varying light and temperature conditions (Zou *et al.* 2011; Chen & Zou 2014). In other brown macroalgae, such as *Fucus serratus*, a down-regulated CCM activity has been reported at elevated CO_2 conditions and it was hypothesized that the energy saving from CCM was used to increase their growth (Johnston & Raven 1991). However, in other *Ochrophyta* with CCM activity, such as *Laminaria digitata* and *Saccharina latissima,* no increase in growth rate was observed when grown at high CO_2 conditions (Roleda & Hurd 2012). These results suggest that algal species may respond differently to increased CO_2 levels. Moreover, the decrease in seawater pH caused by elevated CO_2 may affect other biochemical and physiological processes, such as ion homeostasis, energy budget, respiration, nutrient uptake, extracellular enzymatic activities, as well as early life stages of marine autotrophs (Isreal & Hophy 2002; Rokitta *et al.* 2012; Roleda *et al.* 2012; Hofmann *et al.* 2013).

Most of the effects of elevated CO_2 and OA have been observed in short-term laboratory experiments on a single isolated species. This hampers the scaling up of the observations to predict the impact in natural environments. Indeed, a gene expression study of the coccolithophore *Emiliania huxleyi* revealed that the results of long-term experiments significantly differed from those obtained with short-term exposure (Benner *et al.* 2013). However, studies of the long-term adaptive response of algae to lowered pH and/or high CO_2 are limited to microalgae (Collins & Bell 2006; Lohbeck *et al.* 2012; Benner *et al.* 2013). Investigations of the long-term adaptive response in macroalgae, such as large habitat-forming species, are difficult to perform in the laboratory, due to their longer life cycle, seasonality and

lack of community interactions. In addition, artificial laboratory conditions may cause stress, which could affect the results (Widdicombe *et al.* 2010). Therefore, areas with natural CO_2 vents represent useful experimental systems to investigate the impact of OA on macroalgae in their natural ecosystem. Such sites have been discovered around the globe and few of them have already been used as a "natural laboratory" to study elevated CO_2/OA (Vizzini *et al.* 2010; Lauritano *et al.* 2015; Linares *et al.* 2015).

Off the Ischia Island along Castello Aragonese, natural underwater vents of volcanic origins release gases, mainly CO_2, causing seawater acidification (Hall-Spencer *et al.* 2008). This is one of the best characterized natural CO_2 vent sites in terms of physico-chemical parameters and the presence of autotrophs and associated invertebrate communities (Hall-Spencer *et al.* 2008; Martin *et al.* 2008; Porzio *et al.* 2011; Kerrison *et al.* 2011; Kroeker *et al.* 2013; Porzio *et al.* 2013; Garrard *et al*. 2014; Lauritano *et al.* 2015). Volcanic CO_2 vents acidify the seawater causing a pH gradient with three distinct zones with mean pH of 8.1, 7.8 and 6.7, respectively. In the most acidified area of the vents, the biomass is dominated by fleshy brown algal species, particularly *Sargassum vulgare* (Porzio *et al.* 2011, 2017). *S. vulgare* with its long and complex thallus is a prevalent habitat-forming species along the Mediterranean coast. Its population provides shelter and breeding ground for a large number of associated invertebrate species, mainly molluscs and amphipods (Chemello & Milazzo 2002). This fucoid alga shows synchronous gamete release under calm sea conditions. The negatively buoyant eggs immediately settle down and mating occurs in close vicinity. Moreover, for fucoids and other organisms with direct development, spawning/fertilization and settlement/recruitment processes are tightly coupled (Pearson & Serrão 2006). The dispersal of propagules, gametes and zygotes of many species of fucoid algae is limited to short distance, sometimes within meters, thus

113 limiting the gene flow (Kendrick & Walker 1991). Based on this, it can be assumed that
114 specimens living for decades in our study site with elevated CO_2 and lowered pH conditions
115 could be more isolated and has adopted different mechanisms to persist. Considering that *S.*
116 *vulgare* is a benthic species, its capability to survive in acidified conditions requires fine
117 coordination at the molecular level which is largely unknown due to the lack of genomic
118 information.

119 In order to understand how this macroalga can thrive in lowered pH/high CO_2, we
120 investigated the transcriptional pattern of *S. vulgare* under these conditions. Through a next
121 generation transcriptome sequencing analysis we compared the global gene expression profile of
122 the population from the Ischia Island with the population growing in a control area at current
123 CO_2 and pH levels. To confirm physiological relevance of these transcriptional differences, we
124 measured the activities of selected enzymes.

125 *Sargassum* species, as well as other fucoid algae, provide relevant ecological and
126 economical roles in shallow coastal areas, where they are challenged by increasing
127 anthropogenic pressure. Results from this study will help us to understand the mechanisms used
128 in coping lowered pH environment by habitat-forming macroalgae,

129

130 ## Material and methods

131 ### *Study site and sample collection*

132 The algae were collected along the coast of the Ischia Island at two locations: Castello
133 Aragonese (acidified site, 40°43.87N, 013°57.78E) and Lacco Ameno (control site, 40°45.35N,
134 013°53.13E; Fig. 1). Castello Aragonese has peculiar characteristic features due to CO_2 bubbles

from volcanic vents, resulting in elevated CO_2 levels and lowered pH. Venting activities at this site date back almost 2000 years (Lombardi *et al.* 2011). The vents release gas comprising of 90.1–95.3% CO_2, 3.2– 6.6% N_2, 0.6–0.8% O_2, 0.08–0.1% Ar, 0.2–0.8% CH_4, with no traces of harmful sulfur gases and no rise in the temperature (Hall-Spencer *et al.* 2008). The venting activities are variable at the hour scale, but on average the pH values, where *S. vulgare* occurs, are constantly around 6.7. Almost 6 km northwest from Castello Aragonese, at Lacco Ameno, the unique *S. vulgare* population growing at the same hydrodynamic and physical conditions of the acidified population but at current pH value (8.1) has been found and used as control samples. In both sites *S. vulgare* grows at the same depth (< 1 m depth), in sheltered bays with the same geographical exposure (South-South West). Salinity around Ischia is typical of Tyrrhenian coastal waters and varies generally between 37 and 38.5 psu (Ribera d'Alcalà *et al.* 2004; Lorenti *et al.* 2005; Kroeker *et al.* 2011). The salinity was 37±0.5 ppm at the time of sample collection in March 2014 at both locations. Hence, depth and salinity were not confounding factors. Temperature was continuously monitored using a HOBO data logger (Onset Comp. Corporation, Bourne, MA, USA) which was positioned at Lacco Ameno and Castello Aragonese throughout the year in 2013, and in March 2014. An underwater PAR spherical sensor QSI-140 (Biospherical Instruments, San Diego, USA) was used to obtain noon PAR irradiances and determine *Kd* coefficient from the surface to 1 m depth (Lorenti & De Falco 2004).

In order to avoid the influence of unpredictable and difficult to measure environmental factors between the two locations, the samples were collected at same time point (6th March 2014 at midday). A total of 9 thalli of similar size (8-10 cm frond length) were handpicked in three different patches in each site along a coastal stretch of 15 meters to cover the natural

158 variability of the two local populations. The samples were transported within an hour to the
159 laboratory where apical tissues were cleaned with filtered sea water to remove the visible
160 epiphytes. The samples from each patch were pooled together to create one biological replicate.
161 Samples were then snap frozen in liquid nitrogen and stored at -80 °C prior to RNA extraction
162 and enzymatic assays.
163
164 *RNA extraction and library preparation*
165 RNA was extracted using CTAB extraction method (Sim *et al.* 2013) with minor
166 modifications. The frozen algal tissue was ground to powder in liquid nitrogen and cethyl-
167 trimethyl-ammonium-bromide (CTAB) extraction buffer (100 mM Tris-HCl pH 8.0, 2 M NaCl,
168 20 mM EDTA, 2% (w/v) CTAB, 50 mM DTT, 10% β-mercaptoethanol) was added (1:10 w/v).
169 The mixture was vortexed vigorously and incubated for 10 min at 60 °C followed by chloroform
170 isopropanol extraction (24:1). The supernatant was collected and precipitated with LiCl for 2 h at
171 -80 °C. The pellet collected after centrifugation at 10,000 g for 30 min was washed with 80 %
172 molecular grade ethanol. Total RNA was treated with DNase (Roche) for 20 min at 30 °C and
173 cleaned using RNease plant mini kit (Qiagen). RNA quality and quantity were assessed by
174 NanoDrop (ND-1000 UV-Vis spectrophotometer; NanoDrop Technologies Inc., Wilmington,
175 DE, US), and Plant Nano chip using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa
176 Clara, CA, USA). 2.5 µg of total RNA samples with high purity and high integrity (RIN > 7.5)
177 were used to isolate poly(A) mRNA for the preparation of Illumina RNA-Seq libraries. The
178 libraries were prepared using TruSeq Stranded Total RNA Sample Prep Kit (Illumina Inc., San

179 Diego, CA, USA) and their quality was checked with high sensitivity DNA kit (Agilent
180 Technologies, Santa Clara, CA, USA).
181
182 *De novo transcriptome assembly and data analysis*
183 All 6 libraries (3 from acidified and 3 from control site) were multiplexed and sequenced
184 in a single lane on an Illumina Hiseq 1000 sequencer (Illumina Inc., San Diego, CA, USA)
185 generating 100-bp paired-end sequences. Raw reads were pre-filtered by removing low quality
186 reads (> 50 bases with quality < 7 or > 10% undetermined bases). Adapter sequences were
187 clipped from raw reads using Scythe (version 0.98; https://github.com/najoshi/scythe) and were
188 quality trimmed using Sickle with a PHRED quality score (Q score) minimum threshold of 20
189 calculated in a window of 10 nucleotides (version 0.94; https://github.com/najoshi/sickle). *S.*
190 *vulgare* contigs were assembled *de novo* using Trinity assembler (version 2.0) with default
191 parameters (Grabherr *et al.* 2011) on the two samples separately. Reads were mapped to
192 assembled putative transcripts using bowtie (Langmead & Salzberg 2012). Contigs represented
193 by less than 100 reads, were removed. Redundancy of common transcripts among assemblies
194 was removed by CD-HIT-EST clustering of transcriptome sequences with 95% of sequence
195 identity (Li & Godzik 2006). The threshold was empirically selected as the best in clustering
196 sequences with minimal loss of sequencing data mapping and thus representativeness of the
197 reference dataset. The expression analysis was performed through RSEM (version 1.1.21) using
198 default parameters and expression values have been converted to FPKM (Fragments per
199 Kilobase of exon per Million fragments mapped) (Roberts *et al.* 2011). Differential expression
200 analysis was performed using edgeR software by setting a log fold change on base 2 (LogFC)

201 threshold ≥ 1, corresponding to a 2 fold induction or repression and a False Discovery Rate
202 (FDR) < 0.05 (5%). We performed automatic functional annotation with Blast2GO (Conesa *et al.*
203 2005) and Annocript program (Musacchia *et al.* 2015) using NCBI nr as reference database (E
204 value < 10^{-3}). The Gene ontology (GO) terms were assigned based on annotation with an E-value
205 10^{-6} and 45% cut off followed by InterProScan and KEGG (Kyoto Encyclopedia of Genes and
206 Genomes) pathway analysis in Blast2GO. The regulated transcripts with known functions were
207 also classified into pathways using MapMan (Thimm *et al.* 2004). The WEGO software was
208 used to classify the transcripts according to their functions (Ye *et al.* 2006).
209
210 *Quantitative polymerase chain reaction*
211 To validate the RNA-Seq results, the expression level of 4 genes (2 up- and 2 down-
212 regulated) was measured using quantitative PCR (qPCR). Based on transcriptome analysis, the
213 target genes and 3 reference genes were selected. The list of genes and primers is available in
214 electronic supplementary material (Table S1). Complementary DNAs were synthesized from 1µg
215 total RNA and qPCR reactions were performed with ABI ViiA™ 7 Real-Time PCR System
216 (Applied Biosystems, USA) using FAST START SYBR® green master mix according to
217 manufacturer's instructions. The following thermal profile was used: 95 °C for 10 min, 1 cycle
218 for cDNA denaturation; 95 °C for 1 s and 60 °C for 20 s, 39 cycles for amplification; 1 cycle for
219 melting curve analysis (from 60 °C to 95 °C) to verify the presence of a single product. The
220 qPCR was done in triplicates for each sample. The relative expressions were measured using
221 REST (Relative Expression Software Tool) and statistical significance was calculated using the
222 Pair Wise Fixed Reallocation Randomization Test (Pfaffl 2001; Pfaffl *et al.*2002).

223

224 *Enzymatic activities*

225 Proteins were extracted (Murshed *et al.* 2008) and quantified (Lowry *et al.* 1951) from
226 frozen material. Enzyme activities were determined using existing protocols. Ascorbate
227 peroxidase and monodehydroascorbate reductase were measured according to Murshed *et al.*
228 (2008), peroxiredoxin according to Horling *et al.* (2003), superoxide dismutase according to
229 Dhindsa *et al.* (1981), glutathione S-transferase according to Habig *et al.* (1974), o-aminophenol
230 oxidase according to Suzuki *et al.* (2006), glutamine synthetase according to Temple *et al.*
231 (1996), cellulose synthase according to Wood & Bhat (1988), phosphoglycolate phosphatase
232 according to Ames (1966), cytochrome oxidase according to Goyal & Srivastva (1995). NADH
233 dehydrogenase activity was measured according to Galante & Hatefi (1978) with a modified
234 reaction mixture (1 ml) containing 50 mM phosphate buffer (pH 7.4), 0.1% Triton X-100 (v/v),
235 1.6 mM potassium ferricyanide, 0.17 mM NADH and 30 μg mitochondrial protein in phosphate
236 buffer. This reaction mixture had slightly lower pH value and contained lower and higher
237 concentrations of NADH and potassium ferricyanide, respectively, as compared to the original
238 protocol. Samples treated with L-3,4-dihydroxyphenylalanine (L-DOPA) to suppress NADH
239 dehydrogenase activity were used as negative controls. The absorbance was measured at 410 nm
240 and NADH dehydrogenase activity was calculated using an extinction coefficient of 1 mM^{-1}
241 cm^{-1}.

242

243 *Statistical analysis*

Student tests were performed in order to assess differences between study sites. The enzymatic data were analyzed by using SPSS v21 (SPSS Inc, Chicago, Illinois, USA). Condition of normality was examined by visual inspection and homogeneity of variance by Levene's test. Independent sample t-test was performed on the data to determine the significant difference between the mean values.

Results

Abiotic variables

Temperature at both sites varied annually between 14-28 °C, with warmest water occurring in August and coldest in February and March. Annual means of temperature showed no significant differences between the two study sites ($t=0.96$, $p>0.05$) or between March monthly means ($t=0.084$, $p>0.05$) (Fig. S1). Regarding PAR irradiance, the average *Kd* of the two water masses were similar (0.18 and 0.20 at the acidified and control site, respectively). Therefore we conclude that temperature and light were not confounding factors.

Transcriptomic coverage and annotation results

Illumina based RNA-Seq generated a total of 163,962,462 reads with an average of 25 million of reads per sample (87,870,172 and 76,092,290 reads from acidified and control sites, respectively). 148,262,131 (90.4%) were retained after trimming of low quality reads, and removal of adapter sequences and short reads. These reads were *de novo* assembled for each of the two different geographically isolated populations separately to accommodate for genotype-specific transcript sequences, generating 126,085 contigs representing putative transcripts in

266 control samples and 262,909 in acidified samples. These putative transcripts were filtered to
267 remove background expression noise and redundancy among samples, following which 53,421
268 and 73,253 transcripts remained in control and acidified samples, respectively. A non-redundant
269 reference transcriptome was obtained by combining sequences common to the two datasets
270 (Wachholtz *et al.* 2013; Garcia-Seco *et al.* 2015). The filtered reads were mapped back to the
271 reference transcriptome. An average alignment rate of 77.29% was obtained and a total of 67,131
272 putative transcripts were generated. All raw and processed next-generation sequencing data from
273 this study are available in the NCBI Gene Expression Omnibus under Accession no. GSE78707.
274 The length of these transcripts ranged from 301 to 22,219 bases with an average length of 2,237
275 bases and a median length of 1,906 bases. Out of the 67,131 transcripts, only those with
276 expression values FPKM $\geq$ 0.1 in at least 2 replicates from either of the two conditions were
277 considered to be expressed and used for differentially expressed genes (DEGs) analysis. This
278 filter returned 18,464 putative transcripts for which functional annotation was generated. The
279 average GC content of the *S. vulgare* expressed transcripts were 50.31 % which falls within the
280 range of reported values for *Phaeophyceae* (Le Gall *et al.* 1993). A total of 13,986 (75.75%) was
281 found to have significant hit (E value $< 10^{-3}$) with published sequences. The similarity
282 distribution showed that almost 50% of the hits had a similarity of 40-60%, 40% of the hits a
283 similarity of 60-80%, and nearly 10% of the hits had a similarity of 80% or higher with the
284 known sequences in the database. For almost 80% of the transcripts the first hit was with *E.*
285 *siliculosus* proteins, the only brown alga for which genome sequence was available at the time of
286 data analysis. At least one GO term was assigned to 9,677 transcripts. These transcripts were
287 annotated and GO level 4 terms, based on the principal categories of cellular component (CC),
288 molecular function (MF) and biological process (BP), are shown in Fig. S2. KEGG enzyme

289 codes were assigned to 2841 transcripts representing 119 biological pathways. The highest
290 numbers of transcripts were assigned to primary metabolism, in particular to carbohydrate
291 (21%), amino acid (20%), nucleotide (15%), energy (12%), cofactors and vitamins (10%),
292 followed by lipids (6%) (Fig. S3). There were 111 (0.6%) long non-coding RNAs which are
293 usually involved in transcriptional regulation. These satisfied the following conditions: (i) length
294 200 nucleotides, (ii) lack of similarity with any protein, domain and other short ncRNA from
295 Rfam and rRNAs, (iii) longest open reading frame (ORF) < 100 amino acids and (iv) non-coding
296 potential score > 0.95.
297
298 *Global change of gene expression under acidified conditions*
299 In lowered pH/high CO_2 conditions, 380 transcripts out of 18,464 (2.05%) were
300 differentially expressed, of which 245 transcripts were up-regulated and 135 transcripts were
301 down-regulated (FDR ≤ 0.05). Among all DEGs, 93 showed no similarity to known annotated
302 proteins and 41 exhibited similarity to proteins of unknown functions. Among the transcripts
303 similar to proteins of known functions, 153 were up- and 93 down-regulated. To identify the
304 pathways affected under elevated CO_2/acidified conditions we performed a GO term enrichment
305 analysis using DEGs as test set and whole transcriptome as background in Blast2GO. 32 GO
306 terms were enriched including 21 in BP followed by 7 in MF and 4 in CC ($p \leq 0.05$) (Fig. 2).
307 Over-represented GO categories included generation of precursor metabolites and energy,
308 various cellular and metabolic processes, and transport. The number of the transcripts in each
309 enriched GO term is shown in Table S2. KEGG annotation assigned 98 transcripts (25.7%)
310 belonging to a number of biological pathways (Fig. 3). The most representatives are related to

311 the metabolism of energy (19%), carbohydrates (18%), amino acids (15%), nucleotides (12%),
312 and lipids (12%). KEGG enrichment analysis indicated that oxidative phosphorylation was over
313 represented.
314 To identify relevant mechanisms involved in OA response, we manually classified DEGs into
315 specific groups according to their putative functions: metabolism, ion transport, cellular stress
316 response mechanisms, information storage and processing, membrane protein and cellular
317 signaling (Table S3). An overview of the biochemical pathways affected by OA has been
318 reported in Fig. 4.
319

320 ## Metabolism

321 Among the genes encoding enzymes of the carbohydrate metabolism, fructose-1,6-
322 bisphosphate aldolase was up-regulated, whereas phosphoglycerate mutase (PGM) and triose
323 phosphate isomerase (TPI) were down-regulated. In relation to carbon storage and metabolism,
324 the gene encoding for the enzyme involved in mannitol biosynthesis, e.g. haloacid dehalogenase-
325 like hydrolase (HAD), was up-regulated. A series of transcripts related to cell wall synthesis, e.g.
326 cellulose synthase (CESA), trehalose phosphate phosphatase (TPP), UDP-D-glucuronic acid
327 epimerase (GAE), cell wall associated hydrolase (CWAH), membrane bound sulfotransferase
328 (ST), mannuronan C-5-epimerase (Mc5E) 4, and mannosyl-oligosaccharide alpha mannosidase
329 (MAN) were up-regulated, whereas fucose kinase (FK) and Mc5E were down-regulated.
330 Concerning nitrogen metabolism, the gene encoding the enzyme glutamine synthetase
331 (GS) was down-regulated under acidified conditions.

The up-regulation of the transcripts encoding for mitochondrial electron transport chains and oxidative metabolism genes (FAD linked oxido-reductase, NADH dehydrogenase, cytochrome oxidases) suggest that energy metabolism was upregulated in the *S. vulgare* population at the acidified site.

A total of 16 transcripts encoding alpha, beta and gamma form of carbonic anhydrase (CA) were found in the transcriptome; however, only one α CA was up-regulated. On the contrary, the RuBisCo transcript was not differentially expressed between the populations living at acidified and control sites. Furthermore, several transcripts involved in light harvesting complex and generally photosynthetic process (chlorophyll *a-b* binding proteins, photosystem II S4 domain protein, cytochrome b6f complex iron-sulfur subunit, cytochrome b) and in pigment biosynthesis (1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase) were up-regulated. Also phosphoglycolate phosphatase (PGPase), an important photorespiratory enzyme, was up-regulated in *S. vulgare* population living at the acidified site.

Ion transport

The expressions of a number of transporters involved in ion fluxes as well as in the transport of various metals and metabolites were significantly different between the two sites. These include V-type proton ATPase (V-type ATPase), solute carrier family 35 (SLCF35) and the ABC (ATP binding cassettes) type (ABC), ammonium (NH_4^+T), formate/nitrite (NIR), golgi nucleotide sugar (GONST1), voltage-gated chloride channel (Cl-T), sodium-coupled amino acid (SNAT), and auxin efflux (AEP) transporters.

Cellular stress response mechanisms

Some of the DEGs were involved in cellular stress response such as those encoding for heat shock proteins (HSPs) and antioxidant enzymes. The detected HSPs belonged to two different families: HSP90 and HSP70. For HSP90, 2 transcripts were up-regulated, whereas another transcript and two transcripts coding for HSP70 were down-regulated. Also, the protein associated to the heat shock protein 27 (HSB1), and two other nuclear chaperons (NC) were down-regulated. Moreover, few genes encoding proteins with antioxidant activities were differentially expressed. These included up-regulated transcripts for ascorbate peroxidase (APX), peroxiredoxin (POX), and superoxide dismutase (SOD), and down-regulated transcripts for monodehydroascorbate reductase (MDHAR) and glutathione S-transferase (GST). Other transcripts likely associated with oxidoreductase activity, i.e. cytochrome P450 (cyt P450) and ortho aminophenol oxidase (OAO), were down- and up-regulated, respectively.

Information storage and processing

Several transcripts involved in DNA repair and RNA processing were up-regulated in *S. vulgare* under acidified conditions. Two transcripts encoding histone proteins and three transcripts for structural maintenance of chromosomes (SMC) gene, likely involved in altering gene expression under high CO_2/low pH conditions, were up-regulated. Among transcripts encoding transcription factors, some were up-regulated while others appeared down-regulated.

The expression of several transcripts involved in protein synthesis, including ribosomal structure and biogenesis, was affected. Also some of the transcripts involved in post-translational modifications (PTM) were differentially expressed. In total, the expression of 16 transcripts

encoding elements involved in formation of transposable elements was altered, including 11 up-regulated and 5 down-regulated.

Membrane protein and cellular signaling

Among transcripts involved in cell division, growth and signal transduction, a cyclin A and cyclin B were up-regulated. In addition, Rab/RasGTPase, various kinases, proteases, phosphatases, 14-3-3 proteins, TOR (the target of rapamycin), and some membrane proteins were differentially expressed.

Validation of gene expression analysis

Four genes were chosen to validate the RNA-Seq results by quantitative Real time PCR: phosphoglycolate phosphatase, sterol methyltransferase, ABC transporter and glutamine synthetase. The expression of these genes, normalized using the three reference genes, 60S ribosomal protein, α tubulin, and elongation factor 1alpha, confirmed the RNA-Seq data (Fig. 5).

Enzymatic activities

The activity of selected enzymes involved in stress response, energy metabolism, cell wall synthesis, photorespiration, and nitrogen metabolism were measured to supplement the results of RNA-Seq at a functional level. Consistent with the upregulation of their transcripts, we observed increased activities of superoxide dismutase, ascorbate peroxidase, peroxiredoxin, ortho-aminophenol oxidase, cytochrome oxidase, NADH dehydrogenase, phosphoglycolate phoshatase, and cellulose synthase in samples from the acidified site (Fig. 6). Similarly,

decreased activity of monodehydroascorbate reductase was found consistent with down regulation at gene level. However, activity of glutathione S-transferase and glutamine synthetase differed with gene expression results.

Discussion

In this study, we explored the genome wide transcriptomic response to chronic acidification of a habitat-forming fleshy macroalga, *S. vulgare*, in its natural environment. We generated for the first time large scale genomic information on this macroalga, which could be further used in eco-physiological studies. The sampling was performed once from two locations because the uniqueness of natural CO_2 vents and the lack of other control *S. vulgare* populations at similar environmental conditions did not allow to extend the analysis at a wider spatial scale. However, the number of collected samples along the coastal stretches was high enough to represent the variability of response of local populations. The similarity of the environmental factors between the two sites suggested that the observed molecular response was due to lowered pH/high CO_2.

The analysis of DEGs highlighted the molecular strategies adopted by *S. vulgare* to live at the volcanic CO_2 vents (Fig. 4).

Photosynthetic metabolism

It is commonly believed that increased CO_2 in seawater affects carbon acquisition and fixation. However, our transcriptome data revealed no drastic changes of the related genes in *S. vulgare* at the acidified site. Indeed, only 1 transcript for α-CA was up-regulated and no differences were observed in RuBisCO expression and bicarbonate ion transporters. The reason

for induction of α-CA in high CO_2 compared to ambient CO_2 may be due to a fine regulation of the gene at the higher pressure of CO_2 in acidified site. Assays of CA activity would advance our knowledge in this issue. The increase of a specific type of α-CA (CAH2) was also reported under high CO_2 conditions in *Chlamydomonas* (Spalding 2008). Upon exposure to high CO_2 for long term (250 or 500 generations), no differences in RuBisCO and CA expressions were reported in the microalga *E. huxleyi* (Benner *et al.* 2013; Lohbeck *et al.* 2014). Similarly, expression of small subunit of RuBisCO in the diatom *Thalassiosira pseudonana* was also not altered when grown for a long time under increased CO_2 conditions (Crawfurd *et al.* 2011).

Photorespiration can act as a photoprotective mechanism by diverting the excess excitation energy that may lead to reactive oxygen species formation with consequent damage to biomolecules (Takahashi & Murata 2008). Our finding that both the transcription and the activity of the photorespiratory enzyme phosphoglycolate phosphatase were increased in *S. vulgare* at the acidified site suggests that photorespiration is still active under high CO_2 conditions. However, during photosynthesis, phosphoglycolate phosphatase is also involved in removing inhibitors of Calvin cycle and glycolate metabolism, allowing an increase of carbohydrate production. Noteworthy, in corals a decline in the phosphoglycolate phosphatase was associated with a decrease in the productivity (Anthony *et al.* 2008; Crawley *et al.* 2010).

Considering that in the marine environment photosynthesis is CO_2 limited, a higher number of autotrophs would lead to a better photosynthetic performance in high CO_2 conditions. Indeed, the increase of the transcripts related to light harvesting complex and electron chain of light reactions at the acidified site may indicate the ability to respond to acidification by increasing its energy capture and antioxidative functions (Chen *et al.* 2014).

Carbohydrate metabolism

443 The regulation of the genes involved in the carbohydrate metabolism (fructose-1,6-
444 bisphosphate aldolase, phosphoglycerate mutase and triose phosphate isomerase) indicated the
445 activation of anabolic pathways (gluconeogenesis, Calvin cycle) instead of the glycolytic
446 catabolic ones. Moreover, the up-regulation of haloacid dehalogenase-like hydrolase, which has
447 been shown to have mannitol-1-phosphatase activity in *E. siliculosus* (Groisillier *et al.* 2014),
448 suggested the activation of the pathway leading to the formation of mannitol, one of the main
449 forms of carbon storage in brown algae. Also transcripts related to the formation and structures
450 of cell wall were up-regulated together with the increased transcription and enzymatic activity of
451 cellulose synthase. Overall, these results indicate increased allocation of carbon to cell wall and
452 storage in *S. vulgare*, similar to the response of higher plants to elevated CO_2 (Li *et al.* 2008).

453 **Ion homeostasis**

454 More energy may be needed to maintain the pH homeostasis inside the cells (Rokitta *et*
455 *al.* 2012), which is a critical factor for a range of cellular functions in the algae (Taylor *et al.*
456 2012). Indeed, in *S. vulgare* living at the acidified site there is the up-regulation of V-type
457 proton ATPase which pumps protons across the plasma membrane. In the CO_2-tolerant green
458 microalga *Chlorococcum littorale* an increase in V-type proton ATPase expression was reported
459 when exposed to high CO_2 levels (Sasaki *et al.* 1999). By contrast, no difference in the V-
460 ATPase expression was found in the sensitive green alga *Stichococcus bacillaris* exposed to
461 similar conditions (Dietz *et al.* 2001). This indicated that *S. vulgare* acquired the tolerance to live
462 in high CO_2 conditions. Even if evolutionarily distant from macroalgae, in marine invertebrates
463 such as corals and sea urchins, this transporter was down-regulated in response to OA in short-
464 term experiments (Todgham & Hofmann 2009; Kaniewska *et al.* 2012). In addition to this
465 transporter, the transcript of H^+ transporter ATP synthase, generally involved in pH homeostasis,

466 was constitutively high in both acidified and control sites. The regulation of many transporters
467 involved in ion fluxes, transport of macromolecules and metabolites (chloride, ammonium, ABC,
468 formate/nitrite, sodium coupled amino acid, auxin efflux and solute carrier protein family 35
469 transporters) in *S. vulgare* at the acidified site might be the consequence of the changes in the
470 water chemistry which affects solubility, adsorption, toxicity, and metal redox processes (Millero
471 *et al.* 2009).

472 **Cellular stress response**

473 The antioxidant defense machinery of *S. vulgare* is upregulated, as demonstrated by the
474 increase at transcription and enzymatic levels of superoxide dismutase, ascorbate peroxidase,
475 peroxiredoxin, and ortho-aminophenol oxidase, likely to remove reactive oxygen species
476 deriving from the increased oxidative metabolism. This is further confirmed by the up-regulation
477 of genes involved in oxidative phosphorylation and increased activities of cytochrome oxidase
478 and NADH dehydrogenase, thus supporting the view that *S. vulgare* under acidified condition
479 exhibits an increased energy demand. The finding that the majority of the transcripts encoding
480 HSP90 were up-regulated under acidified conditions is in contrast with the down-regulation of
481 the same genes in the seagrass *Posidonia oceanica* living at Castello Aragonese CO_2 vents
482 (Lauritano *et al.* 2015). On the other hand, the down-regulation of HSP70 observed in *S. vulgare*
483 is in accordance with laboratory experiments mimicking climate change scenarios, in which *E.*
484 *huxleyi* was exposed to acidified conditions for 215 generations (Benner *et al.* 2013). The up-
485 regulation of some antioxidant enzymes and HSPs in the algae at the acidified site further
486 indicates that algae are adopting molecular strategies to thrive in lowered pH conditions.

487 **Information processing**

A general increase in DNA repair, RNA processing, protein synthesis, and post-translational modifications suggests the relevance of these processes in determining tolerance to acidification in *S. vulgare*. Increased translation and post-translational modifications under acidified conditions suggest that this species utilizes the metabolic energy in synthesizing proteins and modifying them to live at high CO_2 conditions (Pan 2013; Pan *et al.* 2015). Interestingly, genes encoding proteins for RNA processing, transcription factors and post-translational modifications were found to be elevated in *E. huxleyi* strains grown under future ocean conditions for more than 200 generations (Benner *et al.* 2013). The regulation of the transcription of histone proteins along with SMC gene and other transcription factors under acidified conditions could be related to the faster growth of *S. vulgare* at the acidified site, likely due to modification of the chromosome structure and altered gene expression (Hennon *et al.* 2015). The presence of the regulated transcripts for enzymes capable of doing transpositions such as reverse transcriptase indicated that the mobile elements may be involved in generating genetic variability at the acidified site which in turn, increases the possibility of adaptation under varying environmental conditions (Capy *et al.* 2000).

Cell signaling

The regulation of membrane proteins and cellular signaling pathways further supports the capacity of the algae to acclimatize/adapt to varying environmental conditions. Briefly, the induction of calcium signaling in acidification response is highlighted by the up-regulation of a specific transcript predicted in MapMan to encode for calmodulin with possible function in thigmomorphogenesis, a complex plant response to abiotic stimuli. Futhermore, the up-regulation of transcripts encoding Rab/RasGTPase and MAPK suggests that MAPK signaling cascades may be also involved in mechanism of response to OA, as also demonstrated in

intertidal macroalgae (Parages *et al.* 2014). The regulation of cyclic adenosine 3',5'-monophosphate (cAMP) in *S. vulgare* may contribute to the acclimatization to elevated CO_2 areas, as also reported for the diatom *T. pseudonana* (Hennon *et al.* 2015). Altogether, the cellular signaling components and membrane receptors affected by decreased elevated CO_2/pH seem to contribute towards growth, division, and development of *S. vulgare* at the acidified site. Indeed, the canopy of this macroalga looks healthy and flourishing at the CO_2 vents of Castello Aragonese.

Conclusions

The results here reported improve our knowledge about the molecular mechanisms underpinning the adaptive response of the brown alga *S. vulgare* to OA. In the future, the identification of the unknown genes revealed by our RNA-Seq analysis should help to further characterize the pathways involved in the survival of *S. vulgare* at the CO_2 site and predict molecular strategies of other fucoid algae growing at the same environmental stress conditions and long-term acclimation to acidification. However, results may differ in other benthic macroalgae for their different carbon uptake strategies. Our results are relevant considering that fucales are endangered species for their high sensitivity to pollution. By contrast, the ability of *S. vulgare* to adapt to acidified conditions at volcanic CO_2 vents suggests that in the future it could be a successful species in a more acidified scenario. This is ecologically relevant because *S. vulgare* is a habitat-forming species contributing to shaping macroalgal communities and trophic dynamics.

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750

751 Data Accessibility

752

753 RNA-Seq raw sequence reads and normalized expression values for each transcript are available
754 through the NCBI Gene Expression Omnibus under Accession no. GSE78707.
755

756 Author Contributions

757

758 AK, IC, FPP, MD, AP, MCB conceived and designed the experiments. AK performed the
759 experiments. AK, IC, AP, MCB analyzed the RNA-Seqdata. HAE, GTSB, HA performed

enzymatic assays. HAE, AK analyzed enzymatic assays. AK, IC, FPP, AP, MCB drafted the paper.

Figure Legends

Fig. 1 Study sites around the coast of the Ischia island: Castello Aragonese (CO_2 vents; acidified site) and Lacco Ameno (control site) (Image generated through QGIS v. 2.12.2).

Fig. 2 Over-represented GO terms under acidified conditions in the three major functional categories, namely biological process (BP), molecular function (MF) and cellular component (CC).

Fig. 3 KEGG biological pathways analysis of differentially expressed genes under acidified conditions.

Fig. 4 An overview of the cellular events involved in the response of *S. vulgare* to acidification at natural CO_2 vents. The differential process and enzymes are shown in green font. Black and red arrow represents up-and down-regulation, respectively. **AA**: Amino acid, **ABC**: ABC transporter, **AEP**: Auxin efflux transporter, **APX**: Ascorbate peroxidase, **CA**: Carbonic anhydrase, **Cdks**: Cyclin dependent kinases, **CESA**: Cellulose synthetase, **Cl^-T**: Chloride transporter, **CWAH**: Cell wall associated hydrolase, **Cyt P450**: cytochrome P450, **ER**: Endoplasmic reticulum, **FK**: Fucose Kinase, **GAE**: UDP-D-glucuronic acid 4-epimerase, **GONST1**: Golgi nucleotide sugar transporter 1, **GS**: Glutamine synthetase, **GST**: Glutathione S-transferase, **HAD**: Haloacid dehalogenase-hydrolase, **HSP**: Heat shock protein, **MAN**: mannosyl-oligosaccharide alpha mannosidase, **MAPK**: Mitogen activated protein kinase, **Mc5E**: Mannuronan C-5-epimerase, **MDHAR**: Monodehydroascorbate reductase, **NC**: Nuclear Chaperons, **NH_4^+T**: Ammonium transporter, **NIR**: Formate/nitrite transporter, **OAO**: o-Aminophenol oxidase, **PGM**: Phosphoglycerate mutase, **PGPase**: Phosphoglycolate phosphatase, **PIP**: Phosphatidylinositol phosphatase, **POX**: Peroxiredoxin, **PTM**: Post-translational modifications, **SLCF35**: Solute carrier protein family 35, **SMC:** Structural maintenance of chromosomes, **SNAT**: Sodium coupled amino acid transporter, **Snrk**: SNF1-related protein kinase, **SOD**: Superoxide dismutase, **ST**: Sulfotransferase, **TF**: Transcription factor, **TOR**: Target of rapamycin, **TPI**: triose phosphate isomerase, **TPP**: Trehalose phosphate phosphatase, **V-type ATPase**: V-type proton ATPase.

Fig. 5 Comparison of gene expression of selected genes by *de novo* transcriptome and real time qPCR. **PGP**: Phosphoglycolate phosphatase, **ABC**: ATP Binding cassette transporter, **SMT**: Sterol methyltransferase, **GS**: Glutamine synthetase.

Fig. 6 Activities of selected enzymes involved in the oxidative stress, energy metabolism, photorespiration, and cell wall synthesis *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

Figure 1

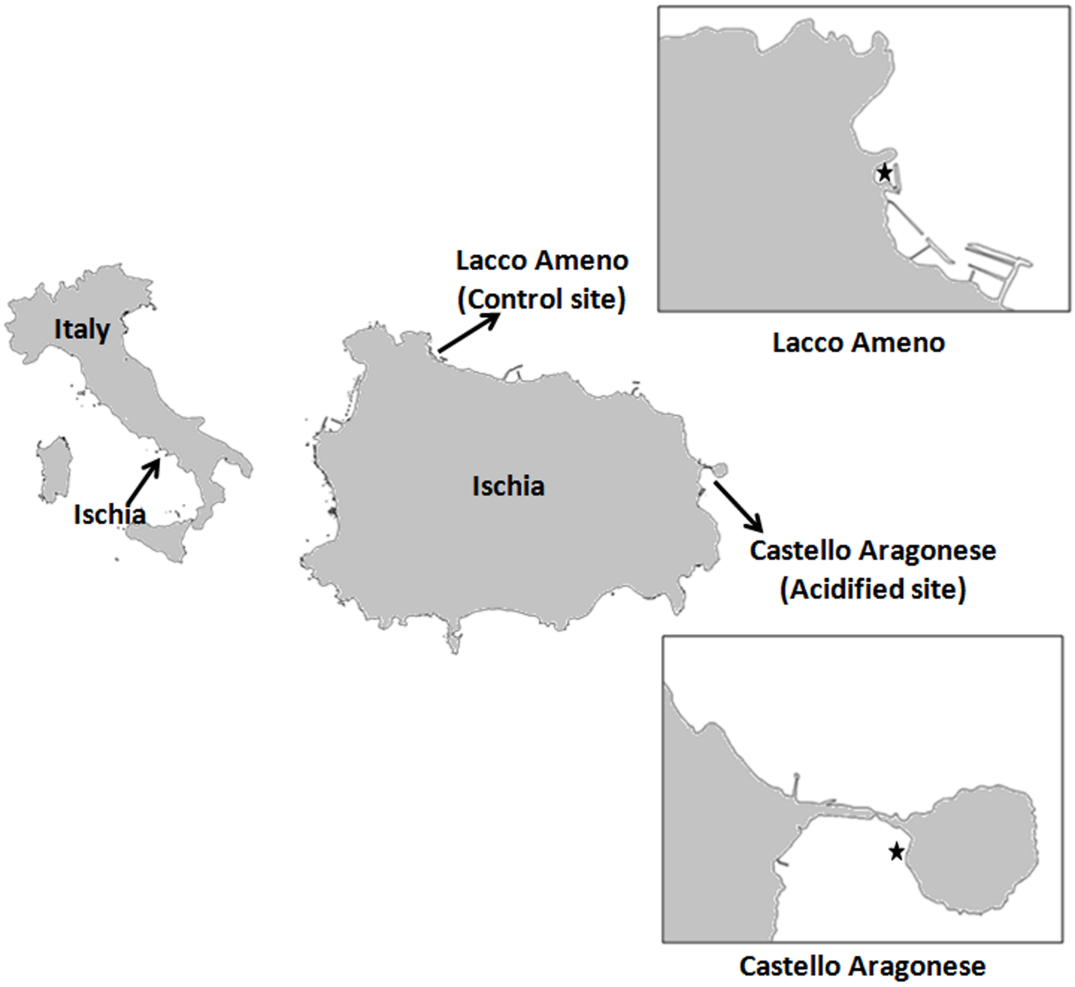

Italy
Ischia
Lacco Ameno
(Control site)
Ischia
Castello Aragonese
(Acidified site)
Lacco Ameno
Castello Aragonese

Figure 2

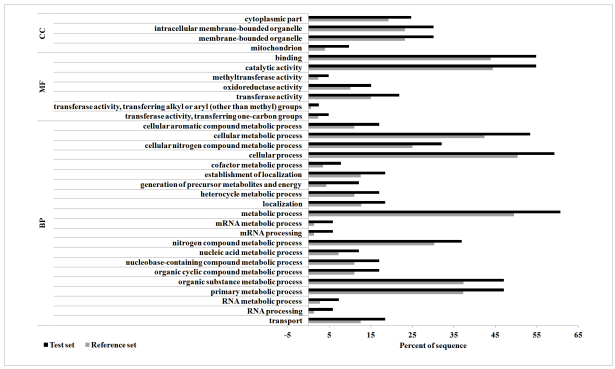

CC
MF
BP
cytoplasmic part
intracellular membrane-bounded organelle
membrane-bounded organelle
mitochondrion
binding
catalytic activity
methyltransferase activity
oxidoreductase activity
transferase activity
transferase activity, transferring alkyl or aryl (other than methyl) groups
transferase activity, transferring one-carbon groups
cellular aromatic compound metabolic process
cellular metabolic process
cellular nitrogen compound metabolic process
cellular process
cofactor metabolic process
establishment of localization
generation of precursor metabolites and energy
heterocycle metabolic process
localization
metabolic process
mRNA metabolic process
mRNA processing
nitrogen compound metabolic process
nucleic acid metabolic process
nucleobase-containing compound metabolic process
organic cyclic compound metabolic process
organic substance metabolic process
primary metabolic process
RNA metabolic process
RNA processing
transport
-5
5
15
25
35
45
55
65
Test set
Reference set
Percent of sequence

Figure 3

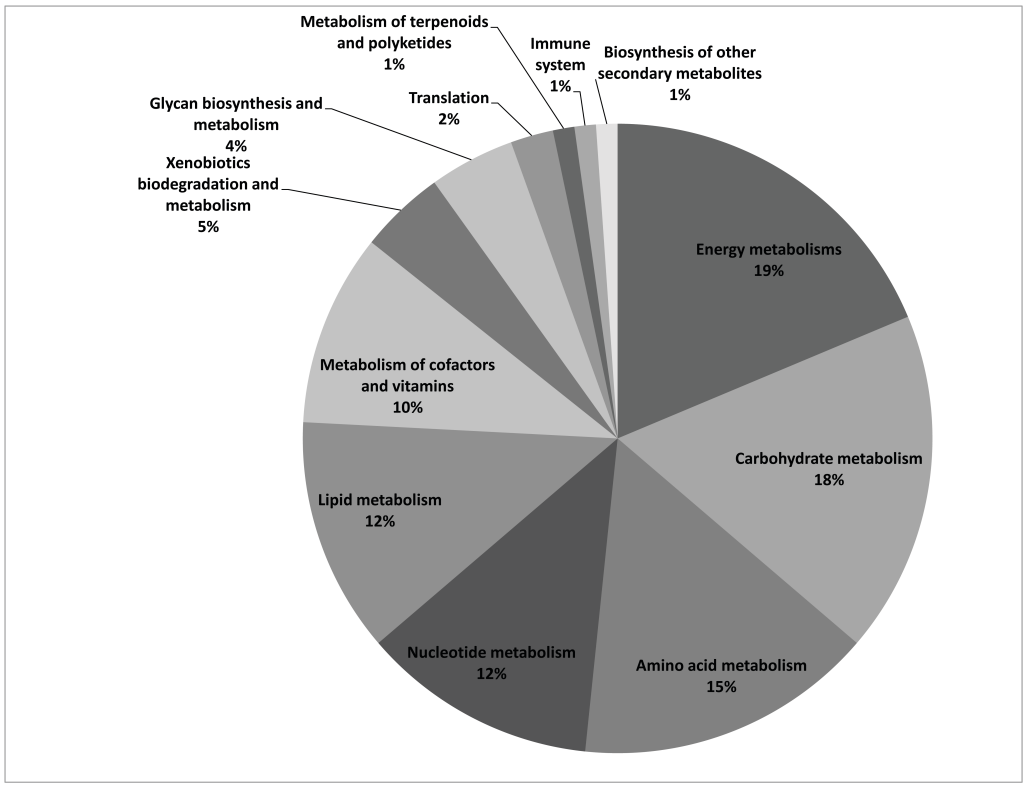

Metabolism of terpenoids and polyketides 1%
Immune system 1%
Biosynthesis of other secondary metabolites 1%
Translation 2%
Glycan biosynthesis and metabolism 4%
Xenobiotics biodegradation and metabolism 5%
Energy metabolisms 19%
Metabolism of cofactors and vitamins 10%
Carbohydrate metabolism 18%
Lipid metabolism 12%
Nucleotide metabolism 12%
Amino acid metabolism 15%

Figure 4

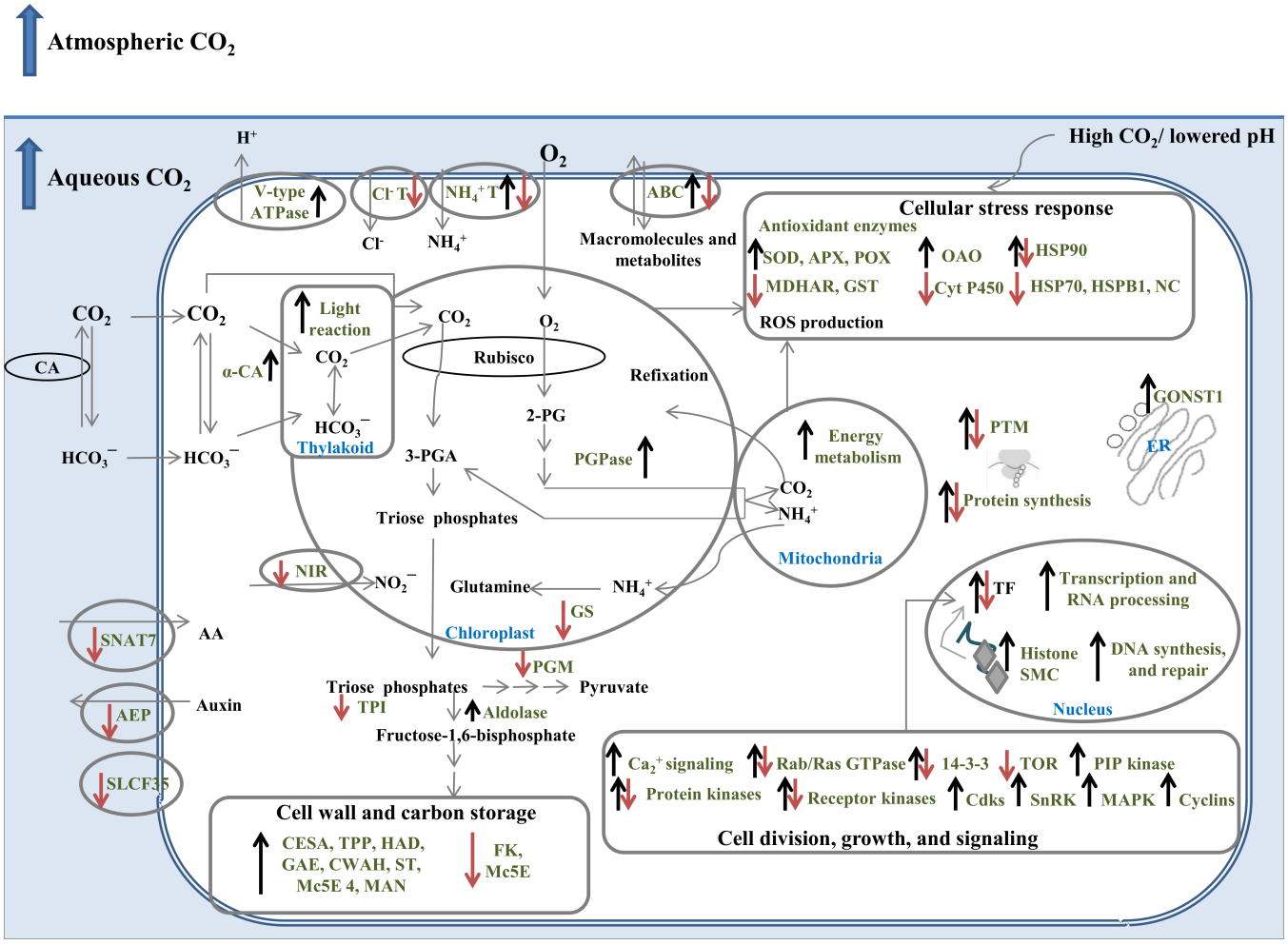

Atmospheric CO_2
Aqueous CO_2
H^+
V-type ATPase
Cl^- T
NH_4^+ T
Cl^-
NH_4^+
O_2
ABC
Macromolecules and metabolites
High CO_2/ lowered pH
Cellular stress response
Antioxidant enzymes
SOD, APX, POX
OAO
HSP90
MDHAR, GST
Cyt P450
HSP70, HSPB1, NC
ROS production
CO_2
CA
HCO_3^-
CO_2
α-CA
Light reaction
CO_2
HCO_3^-
Thylakoid
CO_2
O_2
Rubisco
Refixation
2-PG
3-PGA
PGPase
Triose phosphates
Energy metabolism
CO_2
NH_4^+
Mitochondria
PTM
Protein synthesis
GONST1
ER
NIR
NO_2^-
Glutamine
NH_4^+
GS
Chloroplast
SNAT7
AA
AEP
Auxin
SLCF35
PGM
Triose phosphates
TPI
Aldolase
Pyruvate
Fructose-1,6-bisphosphate
TF
Transcription and RNA processing
Histone SMC
DNA synthesis, and repair
Nucleus
Ca_2^+ signaling
Rab/Ras GTPase
14-3-3
TOR
PIP kinase
Protein kinases
Receptor kinases
Cdks
SnRK
MAPK
Cyclins
Cell division, growth, and signaling
Cell wall and carbon storage
CESA, TPP, HAD, GAE, CWAH, ST, Mc5E 4, MAN
FK, Mc5E

Figure 5

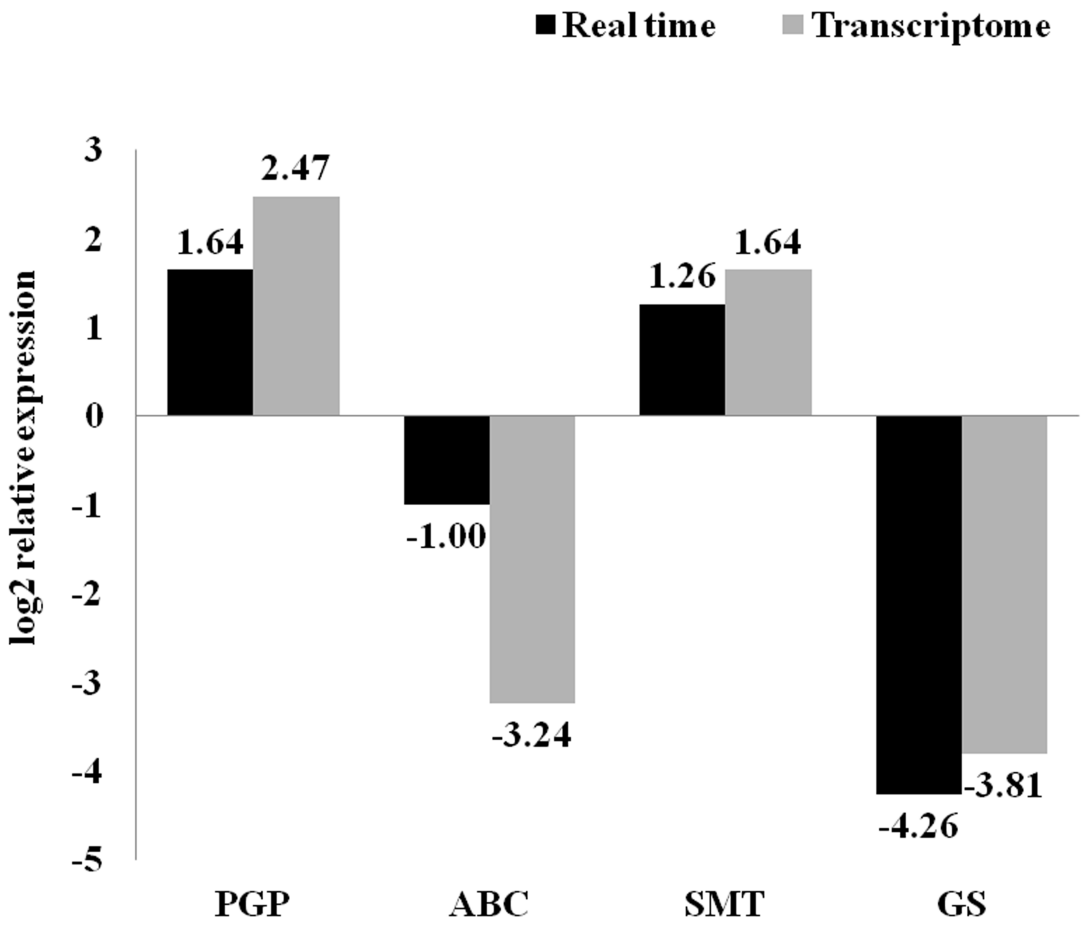
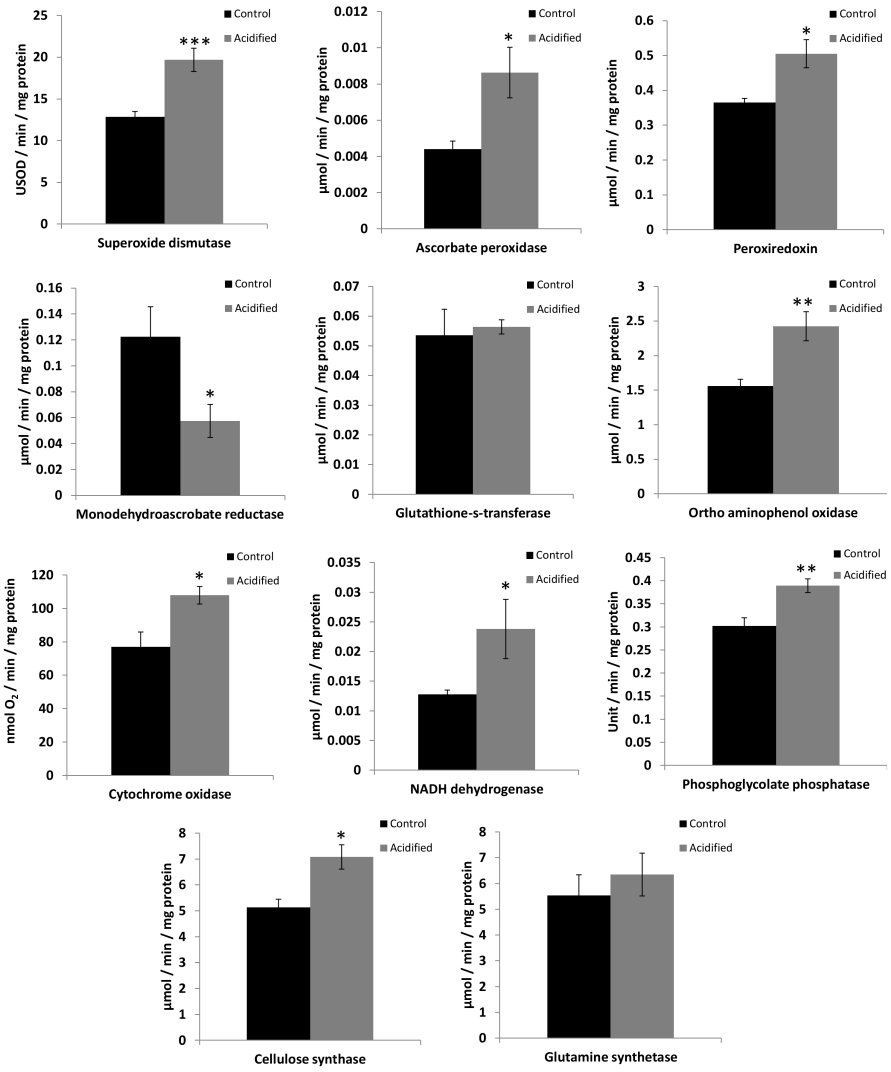
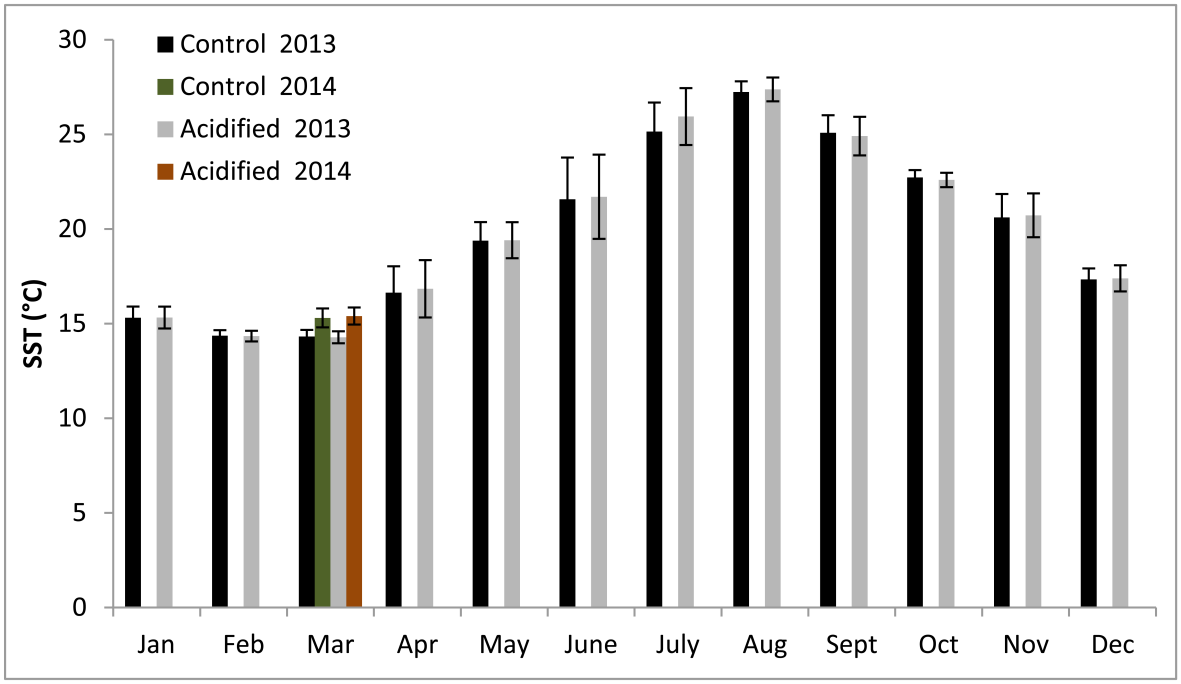

Real time
Transcriptome
log2 relative expression
3
2
1
0
-1
-2
-3
-4
-5
1.64
2.47
-1.00
-3.24
1.26
1.64
-4.26
-3.81
PGP
ABC
SMT
GS

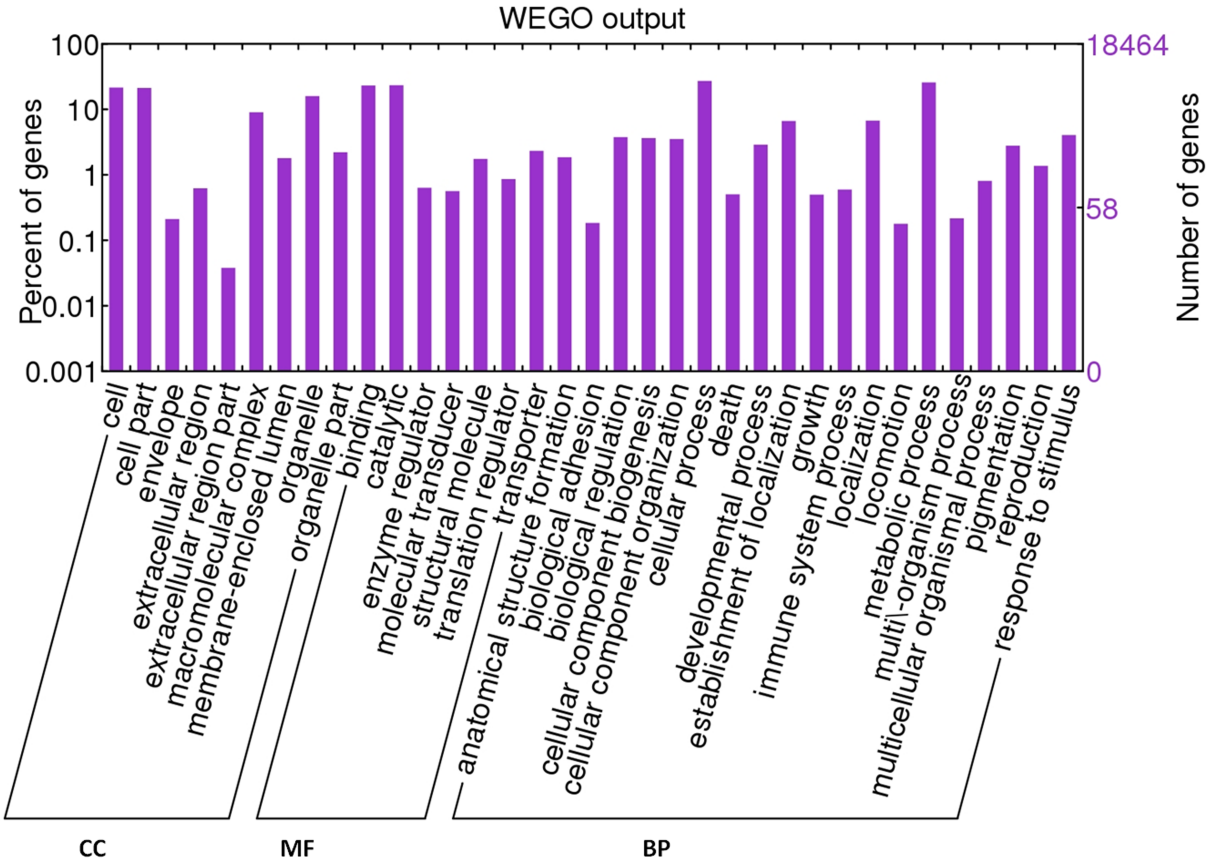
Figure 6


Control
Acidified
USOD / min / mg protein
***
Superoxide dismutase
µmol / min / mg protein
*
Ascorbate peroxidase
µmol / min / mg protein
*
Peroxiredoxin
µmol / min / mg protein
*
Monodehydroascrobate reductase
µmol / min / mg protein
Glutathione-s-transferase
µmol / min / mg protein
**
Ortho aminophenol oxidase
nmol O_2 / min / mg protein
*
Cytochrome oxidase
µmol / min / mg protein
*
NADH dehydrogenase
Unit / min / mg protein
**
Phosphoglycolate phosphatase
µmol / min / mg protein
*
Cellulose synthase
µmol / min / mg protein
Glutamine synthetase

Suppl Fig 1


30
25
20
15
10
5
0
SST (°C)
Control 2013
Control 2014
Acidified 2013
Acidified 2014
Jan
Feb
Mar
Apr
May
June
July
Aug
Sept
Oct
Nov
Dec

Suppl Fig 2


WEGO output
Percent of genes
100
10
1
0.1
0.01
0.001
18464
58
0
Number of genes
cell
cell part
envelope
extracellular region
extracellular region part
macromolecular complex
membrane-enclosed lumen
organelle
organelle part
binding
catalytic
enzyme regulator
molecular transducer
structural molecule
translation regulator
transporter
anatomical structure formation
biological adhesion
biological regulation
cellular component biogenesis
cellular component organization
cellular process
death
developmental process
establishment of localization
growth
immune system process
localization
locomotion
metabolic process
multi\-organism process
multicellular organismal process
pigmentation
reproduction
response to stimulus
CC
MF
BP